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Binding of mannan-binding protein to various bacterial pathogens of meningitis

L. C. VAN EMMERIK, E. J. KUIJPER, C. A. P. FIJEN, J. DANKERT & S. THIEL*
*Reference Laboratory and WHO Collaborating Centre for Bacterial Meningitis, Department of Medical Microbiology, University of Amsterdam and RIVM, Amsterdam, The Netherlands, and *Institute of Medical Microbiology, Aarhus University, Aarhus, Denmark*

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SUMMARY

Mannan-binding protein (MBP), a calcium-dependent plasma lectin, may play a role in the innate defence against microorganisms. After binding to carbohydrate structures at the bacterial surface, MBP activates the classical pathway of the complement system. To investigate the binding capacity of MBP to various bacteria associated with meningitis, an assay was developed to study the binding of MBP to bacteria grown in a semisynthetic fluid culture medium. *Salmonella montevideo* (containing a mannose-rich lipopolysaccharide (LPS)), used as a positive control strain, showed binding of radiolabelled MBP at a level of 80% compared with binding of MBP to zymosan. Binding of labelled MBP to *Salm. montevideo* was time-dependent, temperature-dependent and saturable. The binding was inhibited by unlabelled MBP, by mannose and by N-acetyl-D-glucosamine. Among bacterial pathogens often found to cause meningitis, a wide range of MBP binding capacities could be determined. The encapsulated *Neisseria meningitidis* (representatives from 11 serogroups other than group A were included: $n = 22$), *N. mucosa* ($n = 1$), *Haemophilus influenzae* type b ($n = 10$) and *Streptococcus agalactiae* ($n = 5$) had a low MBP binding capacity of 21·7% (95% confidence interval (CI) 3·3–40·1%). *Escherichia coli* K1 ($n = 11$), *Strep. suis* ($n = 5$), *Strep. pneumoniae* ($n = 10$) and *N. meningitidis* serogroup A ($n = 2$) showed intermediate MBP binding capacity of 58·4% (95% CI 40·0–76·8%). A third group consisting of non-encapsulated *Listeria monocytogenes* ($n = 11$), non-encapsulated *H. influenzae* ($n = 2$), non-encapsulated *N. meningitidis* ($n = 2$), *N. cinera* ($n = 1$) and *N. subflava* ($n = 1$) strains had a high MBP binding capacity of 87·5% (95% CI 62·5–112·5%). The majority of encapsulated pathogens causing bacterial meningitis seem to have a rather low MBP binding capacity.

Keywords mannan-binding protein mannose-binding protein meningitis

INTRODUCTION

In the defence against bacteria causing meningitis, activation of the complement system plays an important role [1]. Inherited or acquired deficiencies of the late complement components (C5–C9) or of components belonging to the alternative pathway (factor D, H, and properdin) predispose for bacterial meningitis, in particular due to *Neisseria meningitidis* [1,2]. The estimated attack rate of meningitis among complement-deficient persons ranges from 45% to 57% [1,3]. The exact mechanism by which an intact complement system is involved in the clearance of bacteria causing meningitis is still unclear.

Correspondence: Dr Ed. J. Kuijper, Department of Medical Microbiology, University of Amsterdam, Academic Medical Center, L-1, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

Mannan-binding protein (MBP), an acute phase serum protein with potential antimicrobial activity, belongs to the calcium-dependent lectins [4–8]. MBP is able to bind to carbohydrate structures present on the surface of bacteria or yeasts [9–12]. MBP bound to carbohydrates activates the complement system leading to a deposition of complement components on the microbial cell wall surface [9,13–15]. MBP exhibits both a structural and functional homology with C1q, the first component of the classical pathway of the complement system, and may represent an important factor in the innate immune defence against microorganisms [16]. Low serum concentrations of MBP have been shown to be associated with an increased risk of microbial infection in infants [17]. Such low serum concentrations seem to exist due to certain point mutations in the gene encoding MBP [18–20]. Interestingly, the serum concentration of MBP has found to be at the

highest level from 6 months to 9 years of age, including a period of life where maternal antibodies have been catabolized and the infant's own adaptive immune system has not yet matured [21].

The purpose of this study was to investigate the MBP binding capacity of various pathogens involved in bacterial meningitis. Since most of the bacterial pathogens of meningitis are encapsulated, the influence of bacterial capsules on MBP binding was also studied using unencapsulated mutants.

MATERIALS AND METHODS

Bacteria

Salmonella montevideo strains 1605 and 5770 (containing mannose-rich polysaccharide subunits of lipopolysaccharide (LPS)), and 3598 (a pmi$^-$ mutant of strain 1605 and unable to incorporate mannose in the polysaccharide chain of the LPS), were a gift of Professor Dr P. H. Mäkelä (Helsinki, Finland). Clinical isolates of neonatal meningitis were *Listeria monocytogenes* serotype 1/2a ($n = 4$), 1/2b ($n = 2$) and 4b ($n = 5$), *Escherichia coli* K1 ($n = 11$) and *Streptococcus agalactiae* subtypes Ib, Ic, III, III/R and non-typable strains ($n = 5$). Isolates derived from young children (< 4 years of age) with meningitis were 24 strains of *N. meningitidis* encompassing two strains each of the serogroups A, B, C, 29E, H, I, K, L, W135, X, Y, and Z, and 10 *Haemophilus influenzae* type b strains. Bacteria causing meningitis in adults were clinical isolates of *Strep. pneumoniae* types 3, 4, 6, 7F, 8, 9N, 9V, 14, 19F and 23F ($n = 10$) and three *Strep. suis* II/R and two R strains. In addition, three non-pathogenic *Neisseriae* species (*N. mucosa*, *N. cinerea*, *N. subflava*), two non-encapsulated variants of *N. meningitidis* serogoup B (strain H4476 and 2996) and two non-encapsulated variants of *H. influenzae* type b (f+ and f0) were included in this study. The clinical isolates and strains were derived from the collection of the Netherlands Reference Laboratory for Bacterial Meningitis. Culturing was at 37°C under mild shaking in 10 ml of semi-synthetic Luria broth (containing 7 mg $(NH_4)_2SO_4FeSO_4.6H_2O$, 50 mg $MgSO_4.7H_2O$, 2 g NH_2Cl, 4·375 g KH_2PO_4, 7·875 g K_2HPO_4, 10 g glucose, 2 g casamino acids per litre and supplemented with 1% isovitalex (Oxoid, Basingstoke, UK). *Haemophilus influenzae* was grown in X- and V-factor (Oxoid) containing Luria broth.

Reagents

D-mannose was obtained from Merck (Darmstadt, Germany); N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), mannosamine, C-reactive protein (CRP), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO).

^{125}I-labelling of mannan-binding protein

MBP and zymosan were isolated as described before [14]. MBP was labelled with ^{125}I using tubes coated with 25 μg Iodogen [22]. The tube was washed twice with TBS (10 mM Tris, 150 mM NaCl, pH 7·4). One hundred microlitres of TBS with 1% glycerol were pipetted into the tube and 10 μl KI of a stock solution of 66 μg/ml in TBS with 1% glycerol and 100 μCi of ^{125}INa (Amersham, Aylesbury, UK) were added. The reaction was started by adding 20 μg of MBP. After 12 min exposure at room temperature, a saturated KI solution was added to end the reaction. The labelled protein was separated from the free ^{125}INa by gel filtration on a 4-ml Sephadex-50 medium column. Before use in the assays, the radiolabelled MBP was applied to an affinity column of TSK-75 beads coupled with mannose using divinylsulphone activation of the beads [23]. After washing the column with TBS with 5 mM $CaCl_2$ pH 7·4, designated as TBS^{2+}, the bound MBP was eluted with TBS with 10 mM EDTA pH 7·4. After recalcification the ^{125}I-MBP was ready for use. The final activity of the ^{125}I-MBP was estimated to be about 1 μCi/μg protein. The ^{125}I-MBP was stored at 4°C with 0·1% BSA added.

SDS–PAGE and autoradiography

To analyse the radiolabelled MBP, SDS–PAGE was performed with a 0·75 mm thick, 4–20% gradient polyacrylamide slab gel under reducing conditions (using 60 mM DTT in the sample buffer) according to the method of Laemmli [24]. Rainbow Markers (Amersham) were used as molecular weight markers. After running the gel, the proteins in the gel were stained with coomassie brilliant blue. An autoradiogram was made by exposing a film to the dried gel for 2 days at −70°C.

Measurements of binding of MBP to zymosan and bacteria

The bacteria were grown to log phase as determined by spectrophotometry at 530 nm and collected by centrifugation at 4000 *g* for 10 min. The bacteria were washed and the number of colony-forming units (CFU) was adjusted to approximately 1×10^{10} CFU/ml (MacFarland 3) with PBS pH 7·4 containing 5% BSA. For each test, 300 μl (approximately 3×10^9 bacteria) of this suspension were pipetted in 5-ml plastic tubes and washed twice with TBS. After the final washing the bacteria were suspended in 300 μl of TBS^{2+} and incubated with ^{125}I-MBP (1000 ct/min) for 1 h at 37°C. The bacteria were pelleted and the pellet was washed with TBS^{2+}. Pellets and supernatants were counted in a gamma-counter (Beckmann) for 10 min. Each binding assay was performed in duplicate, the background radioactivity was subtracted and the mean activity calculated. Binding of MBP to zymosan (this was approximately 700–800 ct/min) was considered as 100% binding, and the binding of MBP to bacteria was expressed as a percentage of this. The same stock of zymosan was used during all experiments. The binding assay with zymosan was carried out in the same way as for the bacteria.

Inhibition experiments

To test whether the labelled MBP behaved as expected, several proteins (unlabelled MBP, CRP and BSA) and carbohydrates were included in the binding assay. The inhibition with EDTA was studied by suspending the bacteria in 50 mM EDTA–TBS buffer before incubation with radiolabelled MBP. In the other inhibition experiments the various proteins and carbohydrates diluted in TBS^{2+} at the indicated concentrations were used to resuspend the bacteria before radiolabelled MBP was added. After this, experiments were continued as described above.

RESULTS

Preparation and control of ^{125}I-MBP

The labelling of MBP with ^{125}I resulted in the incorporation of 1% of the ^{125}I used. Following further fractionation of the labelled MBP by affinity chromatography on mannose-coupled beads, 10% of the labelled MBP was found in the

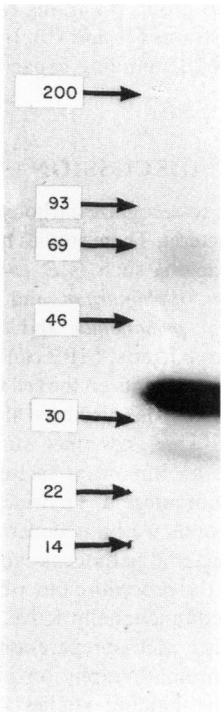

Fig. 1. Autoradiogram of SDS–PAGE at reducing conditions of ^{125}I-labelled affinity-purified mannan-binding protein (MBP). The molecular weights indicated are the molecular weights of the Rainbow markers given by the manufacturer. Radiolabelled MBP is visible as a 32-kD subunit. The faint 26-kD and 65-kD bands probably represent serum amyloid P and dimeric MBP.

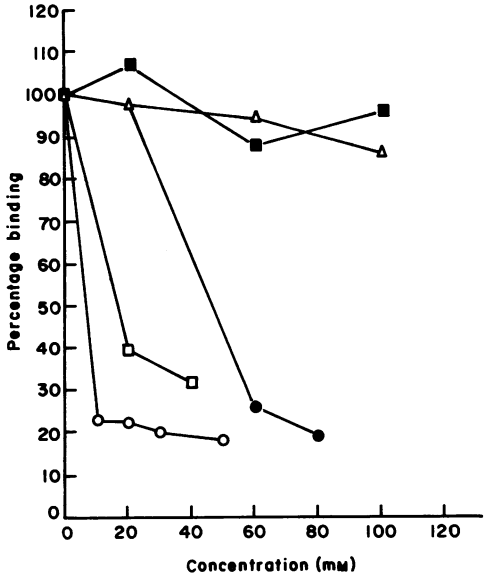

Fig. 2. Effect of EDTA and carbohydrates on the binding of mannan-binding protein (MBP) to zymosan. Labelled MBP was added to zymosan in the presence of EDTA (○), mannose (●), N-acetyl-glucosamine (□), N-acetyl-galactosamine (■) or mannosamine (△).

EDTA eluate. Analysis of the ^{125}I-MBP by SDS–PAGE under reducing conditions revealed a dominant band at 32 kD (Fig. 1). The very faint 26-kD and 65-kD bands seen in Fig. 1 probably represent serum amyloid P component and dimeric MBP, respectively. No further attempts were made to identify these, as the amounts of these proteins in the preparations were negligible (i.e. less than 1%). The binding of ^{125}I-MBP to zymosan was shown to be time- and temperature-dependent. At 37°C, 70–80% of the maximum amount of ^{125}I-MBP was bound to zymosan within 10 min of incubation. The 100% binding was reached after 1 h of incubation, whereas at 4°C this was reached in 4 h. The binding of MBP did not occur in the absence of free calcium ions, as demonstrated by inhibition of the binding by 10 mM EDTA (Fig. 2). The binding could be inhibited by mannose and GlcNAc, but not by mannosamine or GalNAc (Fig. 2). The binding of ^{125}I-MBP to zymosan was diminished by adding increasing amounts of unlabelled MBP, and inhibition was complete when 20 μg of MBP were used. The addition of CRP or BSA had no effect.

Binding of MBP to bacteria

When investigating different growth conditions, it appeared that bacteria grown on agar plates or in liquid medium to stationary phase showed a higher MBP binding capacity than bacteria cultured in liquid medium to the log phase. In addition, the inhibition of the binding of MBP to stationary phase bacteria from agar plates needed higher concentrations of EDTA or mannose than stationary phase bacteria obtained from liquid medium, probably reflecting the more complete carbohydrate structures found in these conditions. To standardize the assay, we used in our experiments only bacteria grown to log phase. Semi-synthetic Luria broth was chosen to avoid any influence of carbohydrates which are present in other culture media. *Salmonella montevideo* was able to bind 80–90% of the ^{125}I-MBP, compared with zymosan. Strains 3598, 1605 and 5770 of *Salm. montevideo* exhibited similar binding capacity (Fig. 3). Addition of EDTA (50 mM), mannose (80 mM) or GlcNAc (80 mM) to the binding reaction showed a

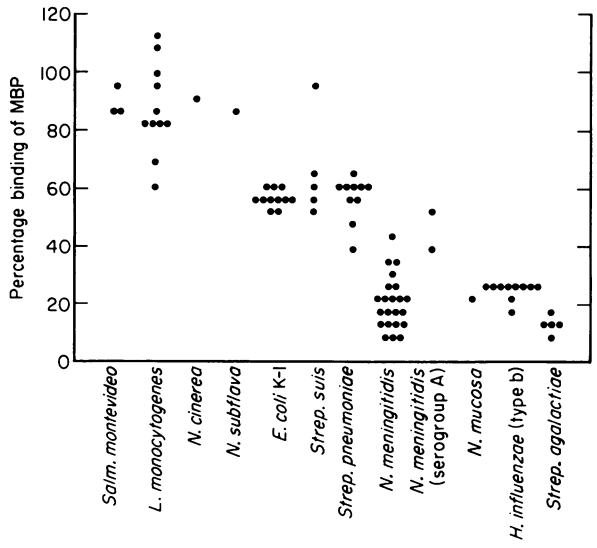

Fig. 3. The binding of mannan-binding protein (MBP) to various bacteria. The binding capacity (expressed as percentage of how much ^{125}I-MBP was bound by zymosan in the same experiment) was calculated from at least two (in most cases three) experiments.

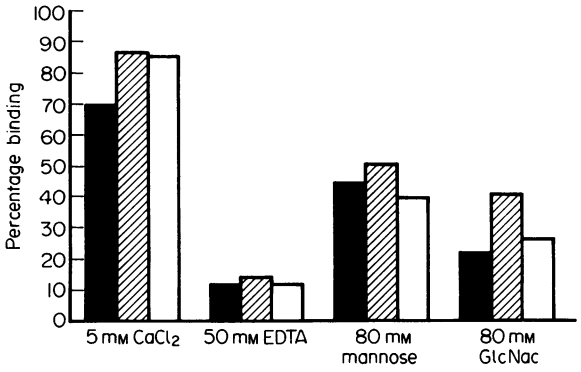

Fig. 4. Effect of EDTA and carbohydrates on the binding of mannan-binding protein (MBP) to three strains of *Salmonella montevideo* (SH 1605 (■), SH 5770 (▨), and SH 3598(□)). The binding is expressed as percentage of how much ^{125}I-MBP was bound by zymosan in the same experiment.

marked decrease of MBP binding (Fig. 4). Addition of mannosamine or GalNAc had no effect (data not shown). We found that all the non-encapsulated strains of *Salm. montevideo*, *L. monocytogenes*, *N. cinera*, and *N. subflava* had a high MBP binding capacity (Fig. 3) compared with zymosan of 87·7% (95% confidence interval (CI) 62·5–112·5%).

There was no difference in MBP binding of the various *L. monocytogenes* serotypes tested. Among the encapsulated strains we identified one group with low MBP binding capacity of 21·7% (95% CI 3·3–40·1%) encompassing *N. meningitidis* (except serogroup A), *N. mucosa*, *H. influenzae* type b and *Strep. agalactiae*, and another group with intermediate MBP binding capacity of 58·4% (95% CI 40·0–76·8%) consisting of *E. coli* K1, *Strep. suis*, *Strep. pneumoniae* and *N. meningitidis* serogroup A (Fig. 3). There was no apparent correlation between influence on the MBP binding capacity and the serotypes within the species *Strep. suis*, *Strep. pneumoniae* and *Strep. agalactiae*. In order to investigate more thoroughly the importance of encapsulation on the binding of MBP, we compared binding to encapsulated and non-encapsulated variants of *N. meningitidis* group B (strains H4476 and 2996) and *H. influenzae* type b (strains f+ and f0). In all strains tested we found a decrease in MBP binding capacity when the capsule was present (Fig. 5).

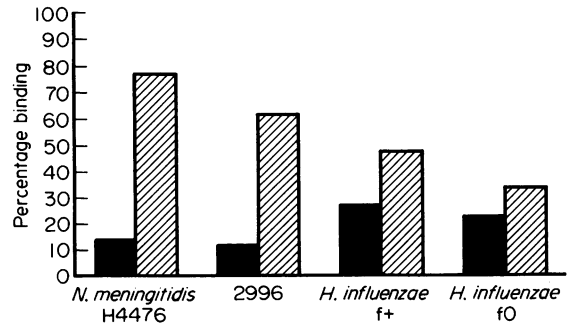

Fig. 5. The binding of mannan-binding protein (MBP) to non-encapsulated variants of encapsulated bacteria. The two *Neisseria meningitidis* strains (H4476 and 2996) and their non-capsulated mutants differed only in the presence or absence of capsules, as is also the case for the two *Haemophilus influenzae* strains (f+ and f0). The binding is expressed as percentage of how much ^{125}I-MBP was bound by zymosan in the same experiment. ■, Encapsulated strain; ▨, non-encapsulated variant.

DISCUSSION

MBP has been shown to recognize carbohydrates present in the cell wall of various bacteria. These studies have so far included a few Gram-negative bacteria such as *E. coli* K12 and J5, *Salm. montevideo* and *Salm. typhimurium* and also Gram-positive bacteria such as *Strep. pneumoniae* and *Strep. agalactiae* [9–12,25]. When bound to bacteria, MBP can promote the deposition of complement components on the cell wall, resulting in lysis of the bacteria [9,12]. MBP has also been shown to behave as an opsonin directly [10]. This and other studies discussed later, indicate that MBP is an important factor in the innate host defence against microorganisms. To widen the knowledge of possible microbial ligands we have undertaken a study of the binding of MBP to bacterial pathogens causing meningitis.

When labelled by the procedure described, 10% of the ^{125}I-labelled MBP remained functionally active. To isolate the active fraction of the labelled MBP preparation the ^{125}I-MBP was purified by affinity chromatography on mannose–TSK beads before performing the binding studies. The finding that a relatively high proportion of ^{125}I-labelled MBP lost its binding capacity suggests that some ^{125}I can bind to a tyrosine residue within the carbohydrate recognizing site of MBP. After concluding the present study, we found that a procedure by which no non-radioactive iodine in the labelling step was used, results in a much higher proportion (between 50% and 60%) of functional active labelled MBP. In order to study further the binding pattern of the labelled MBP, we performed a series of experiments with zymosan as the ligand of choice, since it is known that zymosan has a good binding capacity for MBP [14]. Binding of ^{125}I-MBP to zymosan was calcium-dependent, and was specifically inhibited by the monosaccharides mannose and GlcNAc (Fig. 2). Maximum binding was achieved after 1 h at 37°C, and binding was faster at 37°C than at 4°C. Using the conditions found to be optimal for ^{125}I-MBP binding to zymosan, we then studied the binding of MBP to various bacteria. Two strains of *Salm. montevideo* with mannose-rich LPS bound a high amount of MBP, as was expected. The mutant *Salm. montevideo* strain, with an incomplete o-polysaccharides chain lacking mannose, also showed efficient binding of MBP. This indicates that ligands other than mannose (such as GlcNAc residues) are present in the incomplete LPS of the *Salm. montevideo* strain. The MBP binding to all these strains was specific, as shown by its inhibition by carbohydrates and EDTA.

When we extended our experiments to the binding of MBP to various bacteria causing meningitis, a wide range of MBP binding capacities was found (Fig. 3). One group of non-encapsulated bacteria with high binding capacity included *L. monocytogenes*. The intermediate binding group encompassed *E. coli* K1, *Strep. suis*, *Strep. pneumoniae*, and *N. meningitidis* serogroup A, and the low binding capacity group included *N. meningitidis* (other serogroups than A), *N. mucosa*, *H. influenzae* type b, and *Strep. agalactiae*. Overall, it appeared that the presence of a capsule was associated with a marked inhibition of MBP binding. This points to the capsule as a possible interfering factor for the binding of MBP. Encapsulated *N.*

meningitidis serogroup B (strains H4476 and 2996) and *H. influenzae* type b (f+ and f0 strains) exhibited a low MBP binding in contrast to their non-encapsulated variants. The effect of capsules on MBP binding is further illustrated by the difference of MBP binding capacity between serogroup A and the other meningococcal serogroups. The capsule of serogroup A contains repeating units of partially o-acetylated (1–6)-linked 2-acetamido-2-deoxy-D-mannopyranosyl phosphate groups which is not present in other meningococcal serogroups [26–28]. This serogroup A structure potentially exposes ManNAc, and it seems likely that this is the ligand for MBP (since it is known to be a good ligand for MBP) in the capsular structure [26,27]. The ability of most of the encapsulated bacteria to inhibit the binding of MBP is an addition to the list of properties such as resistance to lysozyme digestion, the inhibition of complement-mediated lysis and the impairment of phagocytosis, all which have been shown to enhance the virulence of encapsulated bacteria [29]. The wide range of MBP binding capacity for different strains belonging to one bacterial species (especially for *L. monocytogenes* and *N. meningitidis*), probably reflects a difference in exposed carbohydrates (e.g. lipopolysaccharides). To study this more thoroughly, we are currently developing assays to measure the binding constants and the number of binding sites.

Recognition molecules of the non-adaptive innate immune system such as MBP appear to play an important role in the onset of infections before the development of an efficient adaptive humoral and cellular immunity, especially in the period of 3 months to 2 years of age, when maternally acquired antibodies are catabolized and the infant's own immune system is still immature. With regard to the ontogeny, MBP rises about two-fold from birth to 3–6 months of age [21]. After 9 years of age MBP levels appear to decline [30]. Thus the concentration of MBP reaches its highest level during the time when the child is most vulnerable to infections. Our data further show that among the bacteria causing neonatal meningitis only *L. monocytogenes* binds MBP to a larger extent. It is possible that a lower level of MBP makes the infant especially vulnerable to this microorganism. This has to be considered in future studies.

It has been found that low concentrations of MBP are associated with a syndrome of frequent unexplained infections in children [17]. The infectious agents in this study were not identified, and there is little information on the type of infections with which we should link MBP deficiencies. Since MBP is only weakly bound to encapsulated bacteria except *N. meningitidis* serogroup A, it is unlikely that subjects with low MBP serum levels or an MBP deficiency are at risk for diseases including meningitis due to these encapsulated bacteria. This hypothesis was confirmed in a recent study, in which no association was found between the occurrence of meningitis due to serogroup B or C and the MBP serum levels [31]. *Neisseria meningitidis* serogroup A, causing epidemic meningitis in certain parts of Africa and China, is found in the Western world as a sporadic cause of meningitis. It will be interesting to study whether these sporadic cases are associated with an MBP deficiency or low serum MBP levels.

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